1641 $

PATENT
1209-0121P

IN THE U.S. PATENT AND TRADEMARK OFFICE

Applicant:	Ulf LANDEGREN	Conf.:	7960
Appl. No.:	08/981,310	Group:	1641
Filed:	December 16, 1997	Examiner:	V. PORTNER
For:	ULTRASENSITIVE IMMUNOASSAYS		

SMALL ENTITY TRANSMITTAL FORM

Assistant Commissioner for Patents
Washington, DC 20231

September 21, 2001

Sir:

Transmitted herewith is an amendment in the above-identified application.

☒ Applicant claims small entity status under 37 C.F.R. § 1.27.

☐ The enclosed document is being transmitted via the Certificate of Mailing provisions of 37 C.F.R. § 1.8.

☐ The enclosed document is being transmitted via facsimile.

The fee has been calculated as shown below:

	CLAIMS REMAINING AFTER AMENDMENT		HIGHEST NUMBER PREVIOUSLY PAID FOR		PRESENT EXTRA	RATE	ADDITIONAL FEE
TOTAL	11	-	20	=		$ 9	$0.00
INDEPENDENT	2	-	3	=		$ 40	$0.00
_____ FIRST PRESENTATION OF A MULTIPLE CLAIM						$135	$0.00
						TOTAL	$0.00

☒ Petition for one (1) month(s) extension of time pursuant to 37 C.F.R. §§ 1.17 and 1.136(a). $110.00 for the extension of time.

☐ No fee is required.

☒ A check in the amount of $110.00 is enclosed.

☐ Please charge Deposit Account No. 02-2448 in the amount of $0.00. This form is submitted in triplicate.

If necessary, the Commissioner is hereby authorized in this, concurrent, and future replies, to charge payment or credit any overpayment to Deposit Account No. 02-2448 for any additional fees required under 37 C.F.R. §§1.16 or 1.17; particularly, extension of time fees.

Respectfully submitted,

BIRCH, STEWART, KOLASCH & BIRCH, LLP

By _____ #32,88
for Gerald M. Murphy, Jr., #28,977

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GMM/MAA/jms
1209-0121P

Attachment

(Rev. 01/22/01)

#25

PATENT
1209-0121P

IN THE U.S. PATENT AND TRADEMARK OFFICE

Applicants:	Ulf LANDEGREN		
Serial No.:	08/981,310	Group:	1641
Filed:	December 16, 1997	Examiner:	V. Portner
Re:	ULTRASENSITIVE IMMUNOASSAYS		

AMENDMENT

Assistant Commissioner of Patents
Washington, D.C. 20231

September 21, 2001

Sir:

In response to the Office Action issued May 21, 2001, the due having been extended for one (1) month to September 21, 2001, Applicants respectfully submit the following remarks.

REMARKS

Rejections under 35 U.S.C.§112, first paragraph and second paragraphs

Claims 1, 3 and 4 have been rejected under 35 U.S.C. §112, first paragraph with the assertion that the specification does not provide support for a genus encompassing kits comprising either three nucleic acids or three cofactors as the first through third affinity reagents. Applicants traverse this rejection and withdrawal thereof is respectfully requested.

The specification on page 4, fifth full paragraph states,

> In Fig. 1 there is shown an immobilized antibody to a specific antigen applied together with two other antibodies, specific for other determinants on the same antigen. Besides antibodies other specifically interacting species with a known affinity, such as lectins, receptors, single chain antibodies, cofactors, oligonucleotides, and other non-proteins, can be used in the invention. *(emphasis added)*

Thus, the specification provides clear support for kits wherein any or all three of the affinity reagents are oligonucleotides or cofactors or any other reagent with a known specific affinity for the protein macromolecule.

In addition, attached hereto is a draft of a manuscript in preparation by the present inventors. The experiments in the manuscript exemplify an assay of the present invention where two of the affinity reagents used in the assay are DNA aptamers and the third reagent is an antisera. The two DNA aptamers recognize independent binding sites on the analyte PDGF (platelet derived growth factor.) Thus, it is possible for the assay of the present invention to have multiple nucleic acids that bind to distinct sites as the affinity reagents. Withdrawal of the rejection is respectfully requested.

Rejections under 35 U.S.C. §112, second paragraph

Claims 3 and 4 have been rejected under 35 U.S.C.§112, second paragraph as being indefinite. More specifically, the Examiner asserts

that it is not clear what three nucleic acids or cofactors might bind to the same protein at the same time. Applicants traverse this rejection and withdrawal thereof is respectfully requested.

As discussed above, it is possible for multiple nucleic acids and cofactors to have distinct binding sites on a protein. As such, the present invention is fully defined as claimed and withdrawal of the rejection is respectfully requested.

Rejections under 35 U.S.C.§102(b)

Claims 1, 3, 4 and 5 have been rejected under 35 U.S.C. §102(b) as being anticipated by Landegren et al. (U.S. Pat. No. 4,988,617). Applicants traverse this rejection and withdrawal thereof is respectfully requested.

> To anticipate a claim, a prior art reference must disclose every feature of the claimed invention, either explicitly or inherently. *See Glaxo Inc.* v. *Novopharm Ltd.*, 52 F.3d 1043, 1047, 34 USPQ2d 1565, 1567 (Fed. Cir. 1995). Hazani v. I.T.C. 44 USPQ2d 1358, 1361 (Fed. Cir. 1997)

The present invention, as encompassed by claim 1, is drawn to, a test kit comprising

a) a first immobilized reagent having affinity to a specific macromolecule, and

b) a second and a third affinity reagent specific for different determinants of said macromolecule, and modified with conjugatable oligonucleotides which conjugate through

i) hybridization of an oligonucleotide complementary to the conjugatable oligonucleotides;

ii) hybridization of the conjugatable oligonucleotides to each other; or

iii) ligation of the oligonucleotides,

wherein a signal is generated by nucleic acid amplification only when said second and third affinity reagents are closely bound on said macromolecule; wherein said macromolecule is a protein.

The assay method of Landegren '617 is "a method of detecting a nucleic acid change in nucleic acids." See the Title and Abstract. Thus, the only "macromolecule" in the assay of Landegren '617 is nucleic acid. The present invention of claims 1 and 3-5 is specifically drawn to a kit having affinity reagents that specifically bind to determinants on a protein macromolecule. The affinity reagents of Landegren et al. '617 are nucleic acids, which specifically bind to other nucleic acids. Thus, the affinity reagents of Landegren et al. '617 do not specifically recognize a protein macromolecule. As such, Landegren et al. '671 does not teach or suggest every feature of the

claimed invention and the present invention is not anticipated by Landegren et al. '617.

The present invention further differs from the disclosure of Landegren et al. because the second and third affinity reagents of the present invention have two separate parts, a first part that specifically binds to the analyte and a second part that is an oligonucleotide that modifies the first part and that is conjugatable.

With Landegren, on the other hand, the affinity reagents have only one part that is an oligonucleotide that binds to nearby portions on the target nucleic acid molecule.

Finally, the present invention differs from Landegren because whereas the second and third affinity reagents of the present invention are closely bound to the analyte to allow the nucleic acid portion of the affinity reagents to conjugate, with Landegren the nucleic acid probes bind to adjacent determinants.

Thus, the present invention is not anticipated by Landegren and withdrawal of the rejection is, therefore, respectfully requested.

Rejections under 35 U.S.C.§102(e)

Claims 1, 3, 4 and 5 have been rejected under 35 U.S.C. §102(e) as being anticipated by Whiteley et al. (U.S. Pat. No. 5,962,223). The Examiner asserts that Whiteley et al. '223 disclose an assay kit with

three nucleic acid affinity reagents, one of which is immobilized. Similar to Landegren et al. '617, the assay of Whiteley et al. '223 pertains to the "detection of specific sequences in nucleic acids." See the Title and Abstract. Thus, the only "macromolecule" in the assay of Whiteley et al. '223 is a nucleic acid. The present invention is specifically drawn to a kit having affinity reagents that specifically bind to determinants on a protein macromolecule. The affinity reagents of Whiteley et al. '223 are nucleic acids, which specifically bind to other nucleic acids. Thus, the affinity reagents of Whiteley et al. '223 do not specifically recognize a protein macromolecule.

Also similarly to Landegren, Whiteley et al. '223 further differs from the present invention because the probes of Whitely et al. '223 are not comprised of two portions, an affinity portion and a nucleic acid that modifies the affinity portion and because the probes of Whiteley et al. '223 also bind to adjacent binding sites.

As such, Whiteley et al. '223 does not disclose every feature of the claimed invention and the present invention is not anticipated by Whiteley et al. '223. Withdrawal of the rejection is, therefore, respectfully requested.

Rejections under 35 U.S.C.§103

Claims 6 and 8-10 have been rejected as being obvious over Cantor et al. (U.S. Pat. No. 5,635,602) combined with Suzuki et al. Claims 1-6 and 8-10 have been rejected as being obvious over Cantor et al. combined with de la Monte et al. (U.S. Pat. No. 5,830,670).

Applicants traverse this rejection and withdrawal thereof is respectfully requested. Cantor et al. discloses an assay where two reagents with affinity for a target protein are connected via double stranded DNA sequences when they are added to a sample. With the present invention the second and third affinity reagents each are connected to a single DNA strand and are separatly added to the immobilized target molecule. With the assay of the present invention, the conjugation of the second and third affinity reagents after binding indicates the juxtaposition of the two target determinants.

With the assay of the present invention, there is almost no background signal because most non-specific binding events will not result in the generation of a signal. At the same time, specific signal can be amplified. These advantages are not possible with the assay of Cantor et al. First, using the assay of Cantor et al. once the one of the one protein becomes immobilized there is an increased risk that the connected protein will also become immobilized even if there is no correct target determinant, increasing the background

signal. Second, in order for the procedure of Cantor et al. to work, all the connected proteins must first be separated using restriction digestion of the oligonucleotide duplex before the oligonucleotides can be amplified. Any non-specifically bound complex that is not cleaved will serve as a template for amplification further creating non-specific background signal. The problems associated with Cantor et al. significantly reduce the assay sensitivity and problems associated with cleaving the connecting oligonucleotide duplex are discussed in column 13, line 51 of Cantor et al. The present invention overcomes the problems of Cantor et al. and as demonstrated by Figure 4 of the attached manuscript the present invention has unexpected, improved sensitivity. There is no suggestion in either Suzuki et al. or de la Monte et al. of modifying Cantor et al. so as to achieve the present invention or of the advantages associated with the present invention.

Suzuki et al. disclose a form of an "immuno PCR" procedure that is disclosed in Sano et al. (1992) Science 258: 120-122. Both Suzuki et al. and the original Sano et al. publication describe that the binding of an antibody to an analyte may be detected using amplification of a nucleic acid sequence added to the antibody bound to the immobilized analyte. With the present invention, however, two separate affinity reagents are required to bind to the immobilized analyte and only when the two affinity reagents are bound to the analyte can the

oligonucleotides be conjugated and a signal generated. As noted above, the assay of the present invention reduces almost all of the background signal created by non-specific binding by requiring that both affinity regents specifically bind before signal is generated. This advantage is not possible with Suzuki et al., nor is this advantage possible with Cantor et al. The attached manuscript compares the present assay procedure, which is dependent on the conjugation by ligation of two nearby oligonucleotides located on one analyte binder, to the assay format encompassed by Suzuki et al. wherein any bound antibody may be amplified. See page 6 and Figure 4. The data demonstrates the advantages associated with the present invention, which are in no way suggested by either Suzuki et al. or Cantor et al. As such, it is not possible to achieve the present invention by combining Suzuki et al. with Cantor et al.

It is further not possible to achieve the present invention by combining de la Monte et al. with Cantor et al. De la Monte et al. does not disclose a new procedure for detecting proteins. The disclosure of de la Monte et al. is directed to a particular class of proteins associated with Alzheimer's disease. The assays disclosed in de la Monte et al. are conventional sandwich ELISA assays for detecting the Alzheimer's proteins. De la Monte et al. disclose in claim 4, the use of three antibodies, however no nucleic acid conjugation or

amplification is disclosed. In claim 6 of de la Monte et al. PCR analysis is disclosed. However, it is evident from column 6, lines 37-43, PCR amplification is contemplated for the detection of only single antibody binding. As such, the procedures disclosed in de la Monte et al. would also have the same background problems discussed above regarding Suzuki et al. and Cantor et al. There is no suggestion in de la Monte et al. of requiring the conjugation between oligonucleotides associated with closely bound probes. Nor is there any suggestion in de la Monte of the unexpected improvements of reduced background and increased sensitivity associated with the present invention. As such, it is not possible to achieve the present invention by combining de la Monte et al. with Cantor et al. and withdrawal of the rejection is respectfully requested.

As the above-presented amendments and remarks in no way add new matter or raise new issues for consideration and further address and overcome the rejections of the Examiner, reconsideration and allowance of the claims are respectfully requested. Should the Examiner have any questions regarding the present application, she is requested to contact MaryAnne Armstrong, PhD (Reg. No. 40,069) in the Washington DC area, at (703) 205-8000.

Pursuant to 37 C.F.R. §§ 1.17 and 1.136(a), Applicant(s) respectfully petition(s) for a one (1) month extension of time for

filing a reply in connection with the present application, and the required fee of $$110.00 is attached hereto.

If necessary, the Commissioner is hereby authorized in this, concurrent, and future replies, to charge payment or credit any overpayment to Deposit Account No. 02-2448 for any additional fees required under 37 C.F.R. §§1.16 or 1.17; particularly, extension of time fees.

Respectfully submitted,

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Attachments

Sensitive Protein Detection via Proximity-dependent DNA Ligation Assays

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The advent of *in vitro* DNA amplification enabled rapid acquisition of genomic information. We present an analogous technique for protein detection, where the coordinated and proximal binding of a target protein by two affinity probes promotes ligation of oligonucleotides linked to each affinity probe. The ligation reaction gives rise to an amplifiable DNA sequence that reflects the presence of the target protein. This proximity ligation assay detects zeptomol amounts of specific proteins without washes or separations, and can be generalized to other forms of protein analysis.

Sensitive and specific methods are required to study the concentration, location and colocalization, as well as secondary modifications of the large sets of specific proteins that are predicted from genome sequence data - stringent demands that cannot be met by current techniques. By contrast, methods in use for detection of specific nucleic acid sequences such as the polymerase chain reaction (PCR) offer excellent sensitivity and specificity (1). This is only partially a consequence of the strong and predictable affinity between complementary nucleic acids strands. Of greater importance is the requirement that pairs of probes recognize a given target sequence, and that only this dual interaction can initiate an amplified detection signal. A requirement for dual and proximate recognition is also a frequent element of cellular recognition reactions in e.g. signal transduction and gene regulation (2).

We have developed an analogous method for *in vitro* detection of proteins and other macromolecules, termed proximity ligation. The method depends on the simultaneous and proximal recognition of target molecules by pairs of affinity probes, giving rise to an amplifiable detection signal. The molecule we targeted was the homodimer of the platelet-derived growth factor B chain (PDGF-BB), a cytokine with growth and differentiation promoting effects (3). As affinity probes we used so-called DNA aptamers – DNA oligonucleotides obtained through a process of *in vitro* selection for affinity to a target molecule (4,5). A DNA aptamer with affinity to PDGF (6) was extended with additional sequence elements at the 5' end or the 3' end, forming a proximity probe pair. When pairs of probes bind PDGF-BB, the free ends of their sequence extensions are brought sufficiently close to hybridize to a subsequently added connector oligonucleotide, allowing the ends to be joined by enzymatic DNA ligation. The action of the detected protein molecules is thus to promote the ligation reaction by bringing together the free ends of the proximity probe pairs. The ligation products can then be replicated by nucleic acid amplification through PCR, while unreacted probes remain silent (Fig. 1).

Based on crude molecular modeling, DNA extensions of the affinity probes of around 40 nucleotides each were selected. However, the length of the extension could be varied over a considerable range with negligible effects on ligation efficiency and nonspecific signal (data not shown). The method thus has the potential also to allow analysis of large

proteins. The connector oligonucleotide was designed to overlap both free ends of the proximity probe pairs by 10 nts, and it was added in a large molar excess over proximity probes to ensure efficient formation of ligation substrates. The excess of connector oligonucleotide also ensured that all proximity probes that failed to form a complex with the target molecule rapidly bound one connector each, rendering such probes unable to undergo ligation, thus minimizing target-independent ligation.

Homogenous assays for PDGF-BB, requiring no washes or separations, were performed by first preincubating proximity probes with the samples for one hour. Next, a mixture was added that included reagents required for probe ligation and for quantitative PCR with real-time detection (7). The samples were then directly transferred to a fluorometric PCR instrument for detection of ligated molecules over a wide concentration range.

To optimize assay parameters a fixed amount of PDGF-BB was analyzed while preincubation volumes and concentrations of proximity probes were varied. All samples were brought to a final volume of 50 µl by the addition of the ligation and amplification mix (Fig. 2A). Increased detection sensitivity was obtained if PDGF-BB-independent ligation events were minimized by using a low concentration of probes, and preincubating the proximity probes and sample in a minimal volume. In further experiments 20 pM proximity probes was used in a preincubation volume of five µl. We estimate that under these conditions the local concentration of ligatable ends of the proximity probes increases by a factor of at least 10^6 upon target binding (8). Neither the signal nor the background increased significantly upon prolonged ligation, consistent with the notion that the excess connector oligonucleotides rapidly bound all 5' and 3' proximity probe ends, blocking further ligation (data not shown).

As little as 24,000 PDGF-BB molecules were reproducibly detected by proximity ligation, approximately 1000-fold less than in a sandwich ELISA for the same protein. The linear range for quantitation extended over a greater than 1000-fold concentration range, with sufficient precision to distinguish two-fold dilutions of the protein (Fig. 2B). Under these conditions, approximately one PDGF molecule in 25 had bound two different proximity probes that were subsequently joined by ligation (9). The aptamers

have a reported 500-fold lower affinity for the PDGF A chain, compared to the B chain, and accordingly the PDGF-AA homodimer was not detected in the assay. Neither was the PDGF-AB heterodimer detected, demonstrating that the assay is truly proximity dependent. The detection reaction could be specifically inhibited through the addition of a fragment of the PDGFß-receptor (10), known to compete with the aptamer for binding to PDGF-BB (6).

The concentration of PDGF-BB in a human serum sample was demonstrated by homogenous proximity ligation to be 0.2 nM (6 ng/ml) (11), compared to a value of 0.13 nM as determined by ELISA. The PDGF-BB content in the corresponding plasma sample was less than 1 pM as determined by ELISA and below 0.2 pM using proximity ligation. The proximity ligation assay further revealed a concentration of 2 pM PDGF-BB in conditioned medium from an eight-day culture of the human anaplastic thyroid carcinoma cell line KAT-4 (12), shown by northern blot analysis to transcribe the PDGF-B gene (N. Heldin, personal communication). This corresponds to an estimated production of 100 molecules per cell and day (13).

Proximity probes were also designed for detection of human alpha-thrombin. A pair of DNA aptamers, binding two distinct sites (13,14), was extended with sequences for ligation and amplification to form a proximity probe pair (Fig. 3). The sensitive detection illustrates that the proximity ligation strategy is applicable when affinity probes for two distinct determinants on one protein are used, and the procedure should therefore also be useful to detect complexes of different proteins.

The homogenous proximity ligation assay is simple to standardize and to perform for large sets of samples. It eliminates the need for extensive, carefully controlled washes, as required in solid-phase assays. However, for samples containing factors that might inhibit ligation or amplification, or where the concentration of analyte is exceedingly low, solid support immobilization of the protein can serve to both purify and concentrate the samples. It also permits removal of unbound probes through washes before the ligation step, further reducing target-independent probe ligation. By immobilizing PDGF-BB via binding to antibody-coated reaction wells, low femtomolar concentrations of the protein were detected using the same pair of proximity probes as was used in the homogenous

assay (Fig. 4). This compares to a low picomolar detection threshold for the ELISA (Fig. 2b).

PCR has previously been used in solid phase protein detection assays for sensitive detection of single affinity probes labeled with DNA strands (immuno-PCR; 15). To compare this strategy with proximity ligation, a single aptamer with an extension amplifiable by PCR was used instead of the ligatable proximity probe pair (Fig. 4). Across a range of probe concentrations this assay resulted in a considerably higher background, and thereby a reduced sensitivity. With an extension, directly amplifiable by PCR, all nonspecifically bound probes can contribute to background signals, while proximity ligation requires proximate binding of pairs of probes in order to yield a signal.

Heteroantisera, monoclonal antibodies, affinity probes prepared by phage/ribosome display techniques (16), or any other affinity reagents should be suitable as proximity probes after being modified through the addition of oligonucleotide extensions. Nonetheless, aptamers have several important advantages in proximity ligation: high-affinity reagents are regularly obtained against a wide range of proteins through a cell-free process that can be automated and scaled up (17), and the attachment of DNA extensions is trivial. Finally, this class of affinity probes is ideally suited to share in the research community by publishing the nucleotide sequence of appropriate reagents, permitting standardization of protein assays between labs.

The proximity-dependent ligation assay converts the detection of any macromolecule for which suitable pairs of affinity probes exist into a more convenient nucleic acid detection reaction. The use of a DNA reporter for the detection reactions permits simultaneous analysis of large sets of proteins by encoding tag-sequences in the amplified segment for separate detection, e.g. by hybridization to a standard oligonucleotide array. Proximity ligation should also enhance the sensitivity and specificity of localized *in situ* detection of proteins, and could provide a valuable means to investigate secondary modifications of specific proteins, as well as the interaction of pairs of biomolecules in macromolecular complexes.

FIGURE LEGENDS

Figure 1. Schematic view of the homodimeric PDGF-BB (18) bound by two aptamer-based proximity probes, A1 and A2, for detection by proximity ligation. The sequence of the aptamer 41t specific for the PDGF B-chain (6), is shown in black, sequence extensions to be joined by ligation upon hybridization to a common connector oligonucleotide (black) are shown in blue (A1) and red (A2), while primer sites for PCR are boxed. A probe for real-time detection of PCR products via the 5' nuclease assay is shown in green with a 5' Fam fluorophore and a 3' Tamra quencher.

Figure 2. Detection of PDGF-BB by proximity ligation. (A) Influence of incubation volumes and probe concentrations in the proximity ligation assay. One µl aliquots, containing 5.6 attomoles PDGF-BB diluted in 137 mM NaCl, 10.1 mM Na_2HPO_4, 1.8 mM KH_2PO_4 pH 7.4, 2.7 mM KCl, 1 mM $MgCl_2$, 1% bovine serum albumin (BSA), were added to ABI optical tubes containing variable volumes of 50 mM KCl, 10 mM Tris-HCl pH 8.3, 3.3 mM $MgCl_2$, 0.1% BSA, and the indicated concentrations of the proximity probes A1 and A2. Upon addition of the ligation and amplification mix the samples contained: 50 mM KCl, 10 mM Tris-HCl pH 8.3, 1.5 mM $MgCl_2$, 0.4 units T4 DNA ligase, 400 nM connector oligonucleotide, 80 µM ATP, ROX internal fluorescence standard, 0.2 mM dNTPs, 0.5 µM primers, 50 nM probe for the 5' nuclease assay, and 1.5 units AmpliTaq Gold polymerase (ABI) in 50 µl. Ligation was completed during transfer of the reactions to the real-time PCR instrument (ABI PRISM 7700). Signal to noise values represent the number of detected ligation products, divided by ligation products arising in a sample without PDGF-BB. The number of ligation products was calculated from a standard curve of diluted amplicons. Averages of duplicate measurements are shown. (B) Dilution series of PDGF-BB analyzed by proximity ligation (1 µl samples) and solid phase sandwich ELISA (100 µl samples; R&D systems). Average values of duplicates/triplicates with s.e.m. are indicated. In the inset panel, the results are shown of analyses of $3x10^{-15}$ mol PDGF-AA, -AB, -BB, and of -BB with 0.3 µM of the PDGF-ß receptor.

Figure 3. Detection of human alpha-thrombin by homogenous proximity ligation. Experimental procedures were identical to those for analysis of PDGF-BB except as indicated below. The proximity probes where Thr1 (5'-*cagtccgtggtagggcaggttggggtgact*cgtggaactatctagcggtgtacgtgagtgggcatgtagcaagagg-3') and Thr2 (5'-P-gtcatcattcgaatcgtactgcaatcgggtattaggcta*gtgactactggttggtgaggttgggtagtcacaaa*-3'), aptamer sequences in italics. The connector oligonucleotide was 5'-aagaatgatgaccctcttgctaaaa-3, and the 5' nuclease probe 5'-TET tgtacgtgagtgggcatgtagcaagagg-3'-TAMRA. Samples were diluted in 100 mM MES buffer pH 6.5 and 1% BSA, and preincubated with proximity probes Thr1 (15 pM) and Thr2 (20 pM) in 100 mM NaCl, 50 mM Tris-HCl pH 7.5, 1 mM $MgCl_2$. The PCR buffer contained 1.9 mM $MgCl_2$. Primers for PCR were 5'-gtgacttcgtggaactatctagcg-3' and 5'-aatacccgattgcagtacgattc-3'. Averages of duplicate measurements are shown with s.e.m.

Figure 4. Dilution series of PDGF-BB analyzed in a solid phase assay, either by proximity ligation (squares) or by aptamer-based immuno-PCR (circles). Five hundred nanogram of an immunoglobulin fraction from an anti PDGF antiserum (19) in 20 µl was added to optical tubes for real-time PCR (Applied Biosystems), followed by blocking with 1% BSA. Up to 200 µl of sample was then incubated together with 5 nM of proximity probes or with 0.1 nM of immuno-PCR reagent. Unbound probes were removed by washes using a multichannel pipette (proximity ligation 8 times with 3 flushes, immuno-PCR 8 times with 4 flushes of 200 µl PBS 0.02% SDS, PBS and finally H_2O). A ligation mix (50 mM KCl, 10 mM Tris-HCl pH 8.3, 1.5 mM $MgCl_2$ 0.15 mM ATP, 50 nM connector oligonucleotide, 2 U T4 DNA ligase, AP-Biotech in 20 µl) was added and the reactions were kept at 30°C for 5 min followed by 20 min at 80°C. Next, a PCR mix was added to a final volume of 30 µl, containing ROX internal fluorescence standard, 0.1 mM dNTPs, 0.2 µM primers, 50 nM probe for the 5' nuclease assay, 0.03 units AmpliTaq Gold polymerase (ABI). The PCR was conducted in an ABI PRISM 7700 Sequence Detection System. The results are represented as averages of duplicate measurements with s.e.m.

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8. By maintaining a very low probe concentration in the ligation phase of the assay, target-independent ligation events (and reagent consumption) are kept to a minimum. However, at very low probe concentration during target binding, too few targets will be bound by a pair of probes, decreasing the signal and the precision of quantification. The increased concentration of proximity probe ends upon protein binding was estimated by assuming that the ends are constrained within a sphere of a radius defined by the size of the protein (≈60 Å) and the length of the fully extended nucleotide extensions (5 Å per nucleotide).

9. The circumstance that not all protein molecules trigger a ligation reaction is probably a consequence of several factors. At 20 pM proximity probe concentrations, well below the reported K_d for the aptamers (approx. 100 pM), not all proteins are expected to have bound two proximity probes. Moreover, only around one in two of such complexes of target protein with two proximity probes involve probe pairs with one 5' and one 3' end available for ligation. Finally, the ligation efficiency of juxtaposed proximity probe ends could be less than 100% due to imperfections of oligonucleotide synthesis, although the fact that prolonged ligation did not yield increased signals indicates that all ligatable ends were rapidly joined.

10. O. Leppänen, *et al.*, *Biochemistry* **39**, 2370 (2000).

11. PDGF-BB was quantified in serum and plasma by adding known amounts of PDGF-BB and calculating the endogenous concentration from the increase in signal. The specificity of the assay was confirmed by blocking probe binding with an excess of

soluble PDGFβ-receptor, bringing the signal to background levels. The ELISA was performed according to the supplier's instructions (R&D systems). The incubation mix also contained 16 μg/ml poly A oligonucleotide and the PCR was performed with 3.5 mM $MgCl_2$.

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13. PDGF-BB expression of the KAT-4 cell line, grown in RPMI supplemented with 10% FCS, was assayed by homogenous proximity ligation of a one μl sample, diluted 5-fold in PBS with 1% BSA, and quantified by reference to analyses of known concentrations of the protein diluted in growth medium. Specificity was confirmed by blocking the signal with an excess of PDGFß-receptor.

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20. Mats Nilsson and Carl-Henrik Heldin offered valuable comments on this manuscript. Olli Leppänen kindly supplied the PDGFß-receptor fragment. The work was supported by the Beijer Foundation and the Technical and Medical Research Councils of Sweden, and by the Swedish Cancer Fund.

FIGURES

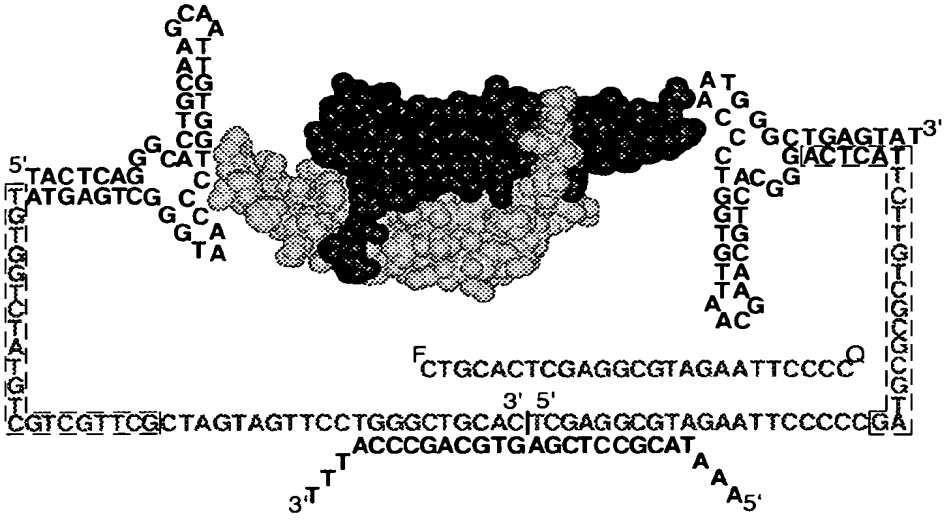

Figure 1.

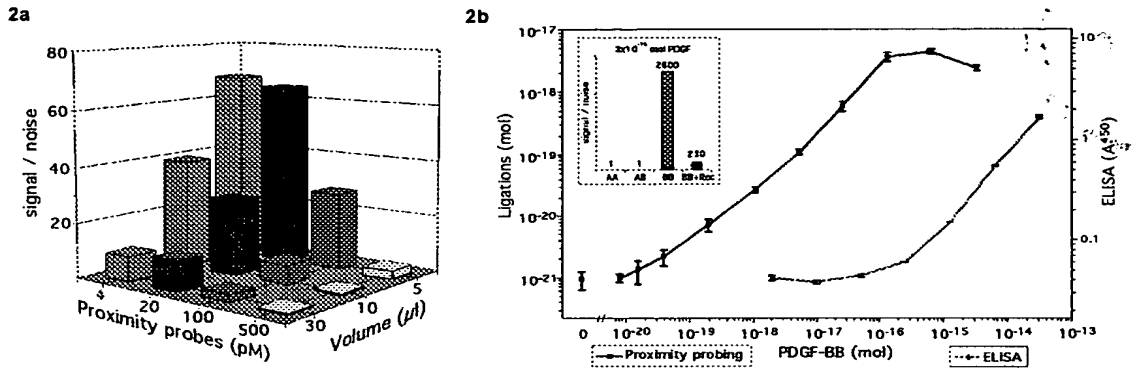
2a
signal / noise
80
60
40
20
Proximity probes (pM)
4
20
100
500
Volume (µl)
30
10
5
2b
Ligations (mol)
ELISA (A450)
PDGF-BB (mol)
Proximity probing
ELISA

Figure 2.

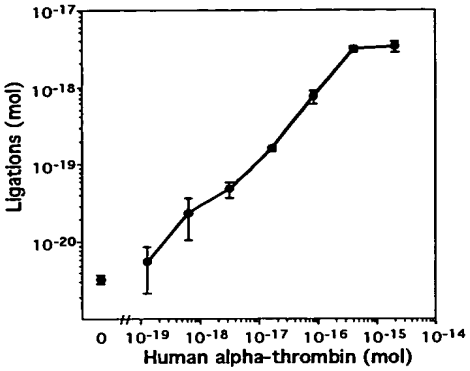
Ligations (mol)
10-17
10-18
10-19
10-20
0
10-19
10-18
10-17
10-16
10-15
10-14
Human alpha-thrombin (mol)

Figure 3.

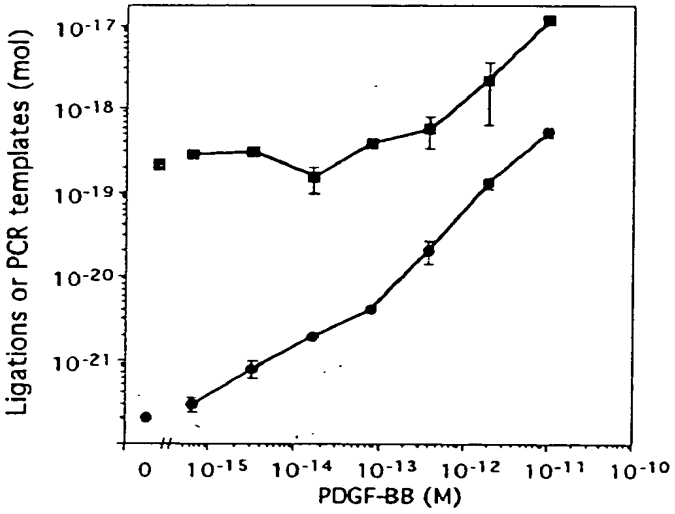
Ligations or PCR templates (mol)
10-17
10-18
10-19
10-20
10-21
0
10-15
10-14
10-13
10-12
10-11
10-10
PDGF-BB (M)

Figure 4.